EU FP VII PROJECT “VITAL”

STANDARD OPERATING PROCEDURE

SOP VITAL 020

Detection and quantification of hepatitis E virus by real-time reverse transcriptase PCR

CREATED:	REVISED:	APPROVED:
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— WARNING —

All samples and controls shall be handled by trained staff in a laboratory with appropriate equipment. Staff must be fully vaccinated against Hepatitis A and poliovirus. Persons using this SOP must be familiar with normal virology laboratory practice. This SOP does not presume to address fully all of the safety issues associated with its use. It is the responsibility of the user to establish appropriate health and safety practices and to ensure compliance with any national regulatory conditions.

AIM

To detect and quantify Hepatitis E virus present in environmental and food samples using quantitative real-time reverse transcriptase (QRT-)PCR.

PRINCIPLE

This protocol is based on the information provided by Jothikumar et al[1].

Standard curves used in QPCR are generated by using serial dilutions of known amounts of RNA Internal Amplification Controls (IACs). Different controls are needed to guarantee the quality of the assay: a NTC (non template control) and an IAC.

For the basic terminology used in this SOP and further information on the principles on which QPCR (TaqMan assay) is based please go to:
http://docs.appliedbiosystems.com/pebiodocs/00105622.pdf

[1] Jothikumar, N.; Cromeans, T.L.; Robertson, B.H.; Meng, X.J., Hill, V.R. 2006. A broadly reactive one-step real-time RT-PCR assay for rapid and sensitive detection of hepatitis E virus. J. Virol. Methods 131: 65–71

EQUIPMENT

- Real-time PCR platform
- Micropipettes of a range of sizes, 1000 µl, 200 µl and 20 µl
- Gloves
- Vortex mixer
- Microcentrifuge
- Refrigerator
- Freezer

REAGENTS AND PLASTICWARE

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles.

- RNA UltraSense™ One-Step Quantitative RT-PCR System (Invitrogen, Catalog Number - 11732-927)
- Tris-EDTA pH 8 (Ambion, Applied Biosystems).
- Nuclease-free water.
- 96-well optical reaction plates (Applied Biosystems. Cat. No. 4306737 for 20 units or 43426659 for 500 units) or similar from other companies.
- Optical adhesive covers (Applied Biosystems. Cat. No. 4311971 for 100 units or 4360954 for 25 units) or similar from other companies.
- Optical adhesive cover starter kit (Cat. No 4313663) or similar from other companies
- Optical caps and their corresponding installing tool (Cat. No 4323032 and 4330015) or similar from other companies.
- Micropipette tips of a range of sizes, 1000 µl, 200 µl and 20 µl

OLIGONUCLEOTIDES

- Forward primer: HEV-F (5'- GGT GGT TTC TGG GGT GAC -3')
- Reverse primer: HEV-R (5'- AGG GGT TGG TTG GAT GAA -3')
- HEV Probe (Taqman probe): HEV-P (5'-FAM- TGA TTC TCA GCC CTT CGC –BGQ1-3')

- IAC MGB TaqMan probe: IACP (5'-VIC- CCA TAC ACA TAG GTC AGG –MGB- NFQ- 3' at a final concentration of 0.100 µM.

* Black hole quencher or non-fluorescent quenchers are strongly recommended instead of TAMRA
MGB: Minor groove binder

PROCEDURE

Standard precautions should be applied in all the manipulations in order to reduce the probability of sample contamination by amplified DNA molecules. Decontaminate the micropipettes after each use. Use UV or cleaning products which can be obtained from your local micropipette suppliers.

NOTE:

The reaction takes place in a 96-well optical reaction plate covered with optical adhesive covers and the optical adhesive cover starter kit. Optical caps and their corresponding installing tool can also be used.

1. Prepare the mix including 0.10× more reaction for inaccuracies during pipetting. Prepare mix in a clean separate area following the tables 1, 2 and 3.

Table 1. Working solutions of primers and probe.
(A starting stock solution of 100 µM for both primers and probes is assumed)

	Stock volume	H_2O	Final volume*	Molarity
Primer HEV-F	25 µl	475 µl	500 µl	5 µM
Primer HEV-R	25 µl	475 µl	500 µl	5 µM
Probe HEV-P	10 µl	490 µl	500 µl	2 µM
Probe IAC	5 µl	495 µl	500 µl	1 µM

* Distribute the final volume solution in 50 µl-aliquots

Table 2. QRT-PCR mix (for one reaction)

Reagent	Working concentration	Final concentration	Volume (µl)
RNA Ultrasense reaction mix	5 ×	1 ×	4.00
Primer HEV-F	5 µM	250 nM	1.00
Primer HEV-R	5 µM	250 nM	1.00
Probe HEV-P	2 µM	100 nM	1.00
IAC probe	1 µM	50 nM	1.00
ROX reference dye	50 ×	1 ×	0.40
RNA Ultrasense enzyme mix			1.00
IAC		*	0.60
Total volume of mix			10
Sample			10
Final volume			20

*see SOP 023 for details on IAC preparation

2. Once the mix has been prepared aliquot 10 µl into each well including the NTC (see Appendix 1). The total volume for one reaction after addition of target will be 20 µl (10 µl mix + 10 µl sample or standard).
3. Add samples (10 µl of the ten-fold dilution of the original sample) in a separate area.
4. Add standard RNA from HEV in duplicate as positive control.
5. Add 10 µl of nuclease-free dd-water in the NTC wells
6. Close with adhesive cover and take care not to touch the cover since finger prints could interfere with the fluorescent signal register by the thermocycler.
7. Perform the QRT-PCR in an real-time PCR platform, selecting the appropriate parameters (considering the use of adhesive cover and the total volume in each well, etc):

Step description		Temperature and time	Number of cycles
Reverse transcription		50ºC for 15 min	1
Preheating		95ºC for 2 min	1
	Denaturation	95ºC for 10 s	
Amplification	Annealing	55ºC for 20ºC	45
	extension	72ºC for 15 s	

8. Once the reaction is completed, store results and data as described in the user's manual of the equipment used.
9. The amount of RNA will be defined as the mean of the data obtained after correcting for the dilution factor (10^{-1}).

APPENDIX 1: CONTROLS

Non-template control (NTC)

The assay must include a NTC to prove mix does not produce fluorescence. This control comprises 10 µl of QRT-PCR mix and 10 µl of nuclease-free dd-water.

Internal amplification control (IAC)

The IAC must be added to every well, to verify that the reaction has worked and has not failed, e.g. through inhibition or instrument failure or operator error. Please see SOP no. 023 for details on the preparation of the IAC.

Contamination

Standard precautions should be applied in all the manipulations in order to reduce the probability of sample contamination by amplified DNA molecules. Decontaminate the micropipettes after each use. Use UV or cleaning products which can be obtained from your local micropipette suppliers.